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Rac1 drives intestinal stem cell proliferation and regeneration

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Adult stem cells are responsible for maintaining the balance between cell proliferation and differentiation within self-renewing tissues. The molecular and cellular mechanisms mediating such balance are poorly understood. The production of reactive oxygen species (ROS) has emerged as an important mediator of stem cell homeostasis in various systems. Our recent work demonstrates that Rac1-dependent ROS production mediates intestinal stem cell (ISC) proliferation in mouse models of colorectal cancer (CRC). Here, we use the adult *Drosophila* midgut and the mouse small intestine to directly address the role of *Rac1* in ISC proliferation and tissue regeneration in response to damage. Our results demonstrate that *Rac1* is necessary and sufficient to drive ISC proliferation and regeneration in an ROS-dependent manner. Our data point to an evolutionarily conserved role of *Rac1* in intestinal homeostasis and highlight the value of combining work in the mammalian and *Drosophila* intestine as paradigms to study stem cell biology.

Keywords: Rac1, intestinal stem cells, *Drosophila*, mouse, ROS, regeneration

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Introduction

Constraining the production of intracellular ROS is critical to maintain homeostatic balance of stem cell-based tissues across species.[1-3] Uncontrolled ROS production leads to stem cell hyperproliferation, misdifferentiation, and loss of regenerative capacity in various systems.[4-6] Consistently, elevated ROS levels are often associated with cancer and aging.[7] However, the cellular and molecular mechanisms leading to ROS production in hyperplastic conditions remain largely unknown.

The intestinal epithelium is constantly replenished by pluripotent ISCs. The remarkable self-renewing capacity of the intestine seems to impact in its high propensity for malignant transformation.[8] ROS are emerging as conserved mediators of intestinal proliferation. Our recent work in the vertebrate intestine shows that ROS is elevated within the proliferative compartment of the intestinal epithelium in a mouse model of CRC driven by loss of the tumor suppressor adenomatous polyposis coli (*Apc*).[9] Treatment with N-acetyl-cysteine (NAC) strongly suppressed intestinal hyperproliferation and stem cell upregulation in *Apc*-deficient mice.[9] Modulation of the redox balance in the adult intestine of the fruit fly *Drosophila melanogaster* directly impacts ISC proliferation. Flies fed with the ROS-inducing compound paraquat or deficient for the ROS-detoxifying enzyme catalase display significant increases in ISC proliferation, leading to intestinal hyperplasia[6,10,11]. On the other hand, treating flies with NAC is sufficient to limit ISC proliferation.[12]

The cellular and molecular regulators of ROS production within stem cells remain largely unexplored. We recently reported that the small GTPase RAC1, which modulates multiple cellular processes and pathways, including ROS production,[13] mediates ROS increase and intestinal proliferation in *Apc*-deficient mice.[9] However, the functional role of RAC1 within stem cells remains to be addressed. Here we use the posterior adult *Drosophila* midgut (**Fig. 1A**) and the mouse small intestine to directly address the role of *Rac1* in ISCs by

means of gain and loss of function experiments. Our results demonstrate that *Rac1* activation in ISCs is necessary and sufficient to drive ISC proliferation and damage-induced intestinal regeneration in an ROS-dependent manner.

Results and Discussion

Rac1 overexpression in ISCs drives ROS production in the adult *Drosophila* midgut

The epithelium of the posterior adult *Drosophila* midgut is replenished by ISCs.[14,15] Each ISC proliferates to give rise to an uncommitted enteroblast (EB), which will differentiate into either an enterocyte (EC) or an enteroendocrine cell (ee). ISCs are the only proliferative cells within the adult fly posterior midgut.

Our recent work shows that deletion of *Rac1* suppresses intestinal hyperproliferation and ROS production in *Apc*-deficient mice.[9] We therefore first asked whether *Rac1* is sufficient to drive ROS production within ISCs in the *Drosophila* midgut. We used the UAS/Gal4 system[16] to specifically overexpress *Drosophila Rac1* in ISCs/EBs (progenitor cells) using the temperature-controlled *escargot-gal4, UAS-gfp; tubulin-gal80^{ts}* driver (*esg^{ts} > gfp*).[14] Overexpression of *Rac1* resulted in a dramatic expansion of the *esg > gfp* cell population and increased ROS production in the midgut (**Fig. 1B–C'**). These results suggest that *Rac1* overexpression in progenitor cells is sufficient to drive ROS production within the intestinal epithelium.

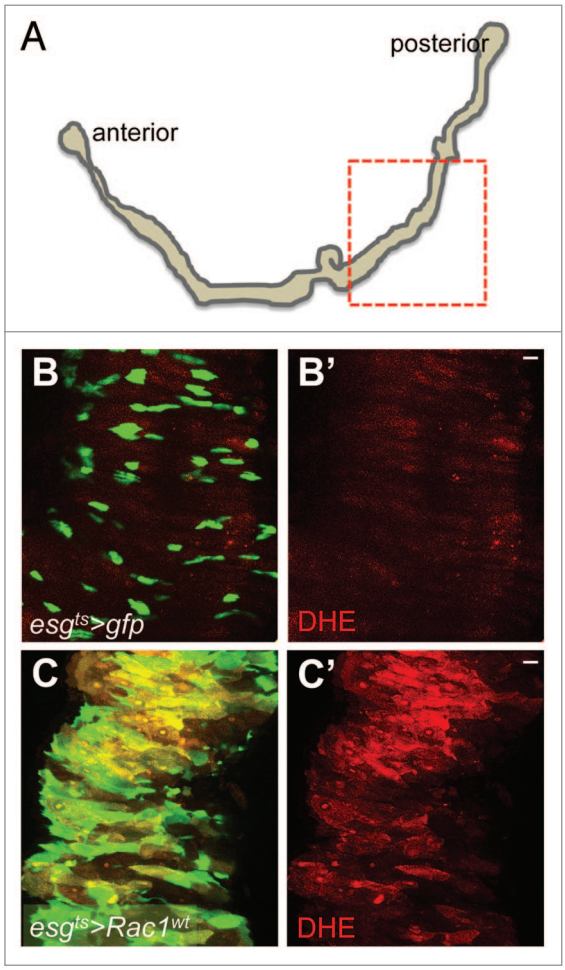

Figure 1. Rac1 overexpression in ISCs drives ROS production in the adult *Drosophila* midgut. (**A**) Tracing of an adult *Drosophila* gastrointestinal tract. The dotted box highlights the region of the posterior midgut, which was used for our studies. (**B–C'**) Posterior midguts of animals incubated at 29 °C during 2 d to induce the expression of *gfp* only (**B and B'**) or *gfp* and *Rac1^{wt}* (**C and C'**) under the control of the intestinal stem cell (ISC)/enteroblast (EB) diver *escargot-gal4* (*esg^{ts} > gfp* and *esg^{ts} > Rac1^{wt}*, respectively). Midguts were dissected and stained with DHE (red) to detect ROS production and anti-GFP (green) to label *esg^{+ve}* cells. Scale bars: 20 μm.

Rac1 overexpression leads to ROS-dependent ISC hyperproliferation in the adult *Drosophila* midgut

The epithelium of the adult posterior *Drosophila* midgut has a remarkable regenerative capacity. Damage induced by agents such as bacterial infection, Bleomycin, or dextran sodium sulfate (DSS) treatment leads to activation of ISC proliferation to regenerate the damaged intestinal epithelium.[12,17-19] Previous work demonstrated that ROS production is essential for damaged-induced ISC proliferation in the fly midgut.[12] We therefore asked whether ROS upregulation was important for the phenotype resulting from *Rac1* overexpression in the midgut. Consistent, with the previous report[12] preventing ROS production by NAC impaired ISC proliferation in posterior midguts from flies infected with the pathogenic bacteria *Pseudomonas entomophila* (*Pe*) (**Fig. 2A–C' and F**). Importantly, NAC treatment strongly suppressed ISC hyperproliferation in *Rac1*-overexpressing midguts (**Fig. 2D–E' and F**). These results suggest that ROS production is essential for *Rac1*-dependent ISC hyperproliferation in the intestine.

Rac1 is required for intestinal regeneration in the *Drosophila* and mouse intestine

We finally asked whether *Rac1* was necessary to drive ISC proliferation in response to damage. This is a question, which also derives from our previous work in the mammalian intestine.[9] We used the genetic approach described in **Figure 1** to knockdown *Rac1* within progenitors cells of the *Drosophila* midgut by RNA interference (RNAi) (*esg^{ts} > Rac1-IR*). Knockdown of *Rac1* by 2 independent RNAi lines (**Fig. 3B–C'**) resulted in almost complete suppression of ISC proliferation in regenerating posterior midguts subject to *Pe* infection (**Fig. 3B–D**; compare with **Fig. 3A, A', and D**). Similar to the *Drosophila* midgut, the mammalian intestine displays a remarkable regenerative capacity following damage.[20] We therefore addressed if the requirement for *Rac1* during intestinal regeneration is conserved across these species. We conditionally deleted *Rac1* from the mouse intestinal

epithelium using the *vil-Cre-ERT2* and tested the effect of *Rac1* loss on tissue regeneration upon DNA damage (see "Materials and Methods"). Consistent with our results in the fly midgut, *Rac1* deletion significantly suppressed regeneration in the mouse intestinal epithelium (**Fig. 3E–G**).

Altogether, our results suggest a central conserved role for the small GTPase RAC1 as a driver of ISC proliferation through the production of ROS. These data highlight RAC1 as key player and potential therapeutic target for conditions linked to oxidative stress such as cancer and aging.

Materials and Methods

Fly stocks

esg^{ts} > *gfp* (Shigeo Hayashi)
UAS-Rac1^{wt} (Bloomington stock: 6293)
UAS-Rac1-IR^{TRIP} (Rac1 RNAi) (Bloomington Stock: 34910)
UAS-Rac1-IR^{VDRC} (VDRC stock: 49246)

Fly maintainace and genetics

Crosses were maintained at 18 °C in standard medium. Only posterior midguts of female flies were analyzed in this study (**Fig. 1A**). Animals of the desired genotypes were collected within 48–72 h of eclosion, and then switched to 29 °C incubators to activate the expression of the desired transgenes under the control of *gal4/gal80*^{ts}. Fly food was changed every other day. The phenotype of *Rac1* overexpression was analyzed 2 d after transgene activation. The requirement of *Rac1* during intestinal regeneration was assessed following 10 d of RNAi overexpression.

Fly Genotypes

yw; escargot-gal4, UAS-gfp/+; tub-gal80^{ts}/+
yw; escargot-gal4, UAS-gfp/UAS-Rac1^{wt}; tub-gal80^{ts}/+
UAS-Dicer2/+; escargot-gal4, UAS-gfp/+; tub-gal80^{ts}/ UAS-Rac1-IR^{TRIP}
UAS-Dicer2/+; escargot-gal4, UAS-gfp/ UAS-Rac1-IR^{VDRC}; tub-gal80^{ts}/+

Mouse genotypes

All experiments were performed under the UK Home Office guidelines. Experiments were performed on mice of mixed background (50% C57Bl6J, 50% S129). The alleles used were: *vil-Cre-ERT2*[21] and *Rac1*^{fl}.[22]

Histology and tissue analysis

Immunofluorescence

Tissues were dissected in PBS and fixed 30–45 min in 4% para-formaldehyde (Polysciences, Inc). After fixation, samples were washed 3 times in PBS + 0.1% TritonX-100 (PBST) and incubated in primary antibodies over night at 4 °C. Samples were then washed as described and subjected to secondary antibody staining for 2 h at room temperature followed by washing and mounting on Vectashield containing DAPI (Vector Laboratories, Inc). Primary and secondary antibodies were incubated in PBST+ 0.5% BSA. Primary antibodies used: chicken anti-GFP 1:4000 (Abcam); rabbit anti-pH3 S10 and S28 1:100 (Cell Signaling). Secondary antibodies used: Alexa 488 1:200 and Alexa 594 1:100 (Invitrogen).

Confocal images were captured under a Zeiss 710 Confocal microscope using 20X (**Fig. 3**) and 40× (**Figs. 1 and 2**) lenses and 1.0 optical zoom. Images were processed with Adobe photoshop CS.

Detection of ROS by DHE staining

DHE staining was done as described in ref. 6. Briefly, fly guts freshly dissected in Schneider medium (HyClone) were incubated in medium containing 30 μM DHE (Invitrogen) for 5 min at room temperature and protected from light. Tissues were then washed, mounted, and immediately imaged.

Tissue analysis of mouse small intestines

Standard histological techniques were used throughout. Tissue was fixed in 4% neutral buffered formalin and stained with hematoxylin and eosin. Slides were analyzed using an Olympus BX51 microscope and images taken with a DP70 camera.

Fly feeding experiments

Pe feeding experiments were done as previously described.[23] Briefly, flies were subject to starvation by placing them in empty vials at 29 °C for 2 h prior to feeding them the different

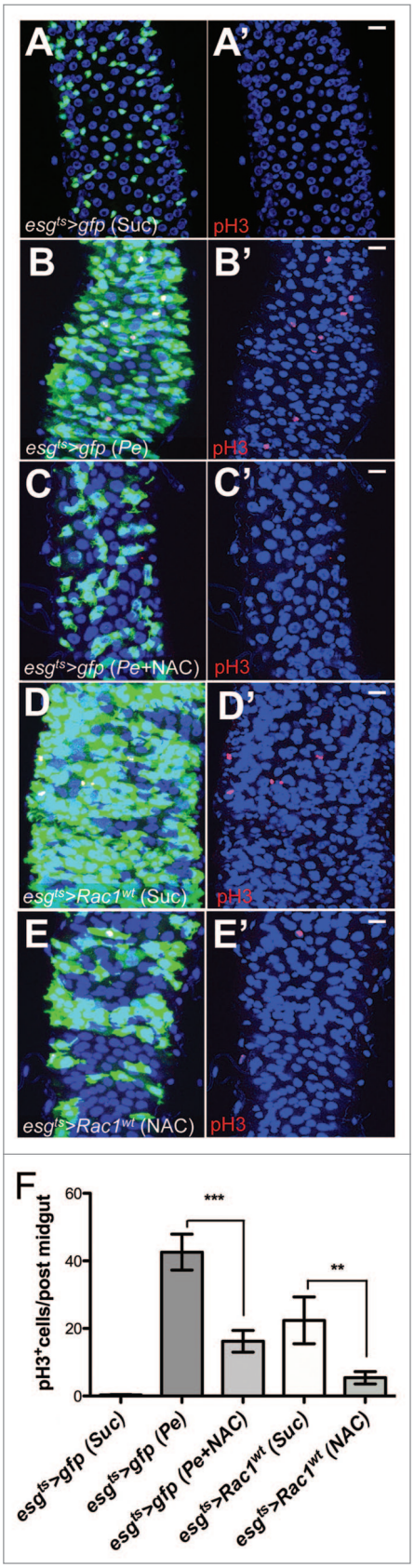

Figure 2. Rac1 overexpression leads to ROS-dependent ISC hyperproliferation in the adult *Drosophila* midgut. (**A–E'**) Posterior midguts from *esg*^{ts} > *gfp* and *esg*^{ts} > *Rac1*^{wt} animals fed with Sucrose (**A, A', D, and D'**); *Pe* (**B and B'**); *Pe* + NAC (**C and C'**), or NAC only (**E and E'**). Midguts were dissected and stained with anti-GFP (green; left panels) to label *esg*$^{+ve}$ cells and anti-pH3 to visualize proliferating ISCs (red; right panels). DAPI (blue) labels all cell nuclei. (**F**) pH3 counts of posterior midguts of animals as in (**A–E'**) (***$P < 0.0001$; ** $P < 0.001$ one-way ANOVA with Bonferroni multiple comparison test). Scale bars: 20 μm.

agents. Animal were then kept at 29 °C and fed either 5% sucrose (Suc); 10× overnight *Pseudomona entomophila* culture in 5% sucrose (*Pe*); NAC (10 mM final concentration in 5% sucrose; Sigma) (NAC); or 10× overnight (*Pe*) + NAC (10 mM final concentration) (*Pe* + NAC) on filter-paper discs (Whatman). NAC treatment was done for 2 d, and *Pe* infection was done for 1 d during the last day of incubation. Media were changed daily. Guts were then dissected and analyzed using immunofluorescence and confocal imaging. *esg*^{ts} > *Rac1*^{wt} animals were treated with Sucrose or NAC during transgene activation.

Mouse intestinal regeneration

Conditional deletion of *Rac1* was induced by single intraperitoneal injections of 80 mg/kg tamoxifen on 2 consecutive days. Intestinal regeneration was induced by irradiating mice with 8 Gy gamma-irradiation the day after the first tamoxifen injection. Mice were sacrificed 72 h post-irradiation and the small intestine isolated and flushed with tap water. 10× 1 cm portions of small intestine were bound together with surgical tape and fixed in 4% neutral buffered formalin. Hematoxylin and eosin stained sections were used for analysis. Crypts were scored as regenerating if they contained 6 or more consecutive cells. The average number of regenerating crypts across cross sections of the 10 gut pieces was used for statistical analysis. Three control and four *Rac1*-deleted mice were used for this experiment.

Quantifications and statistics

We analyzed between 5–10 posterior fly midguts and 3–4 mouse small intestines in each experiment. Quantification of the data was presented in bar graphs created with Graphpad Prism 5. Data represents average values ± SEM. We used one-way ANOVA with Bonferroni multiple comparison test and unpaired *t* test to calculate statistical significance in our fly and mouse experiments, respectively.

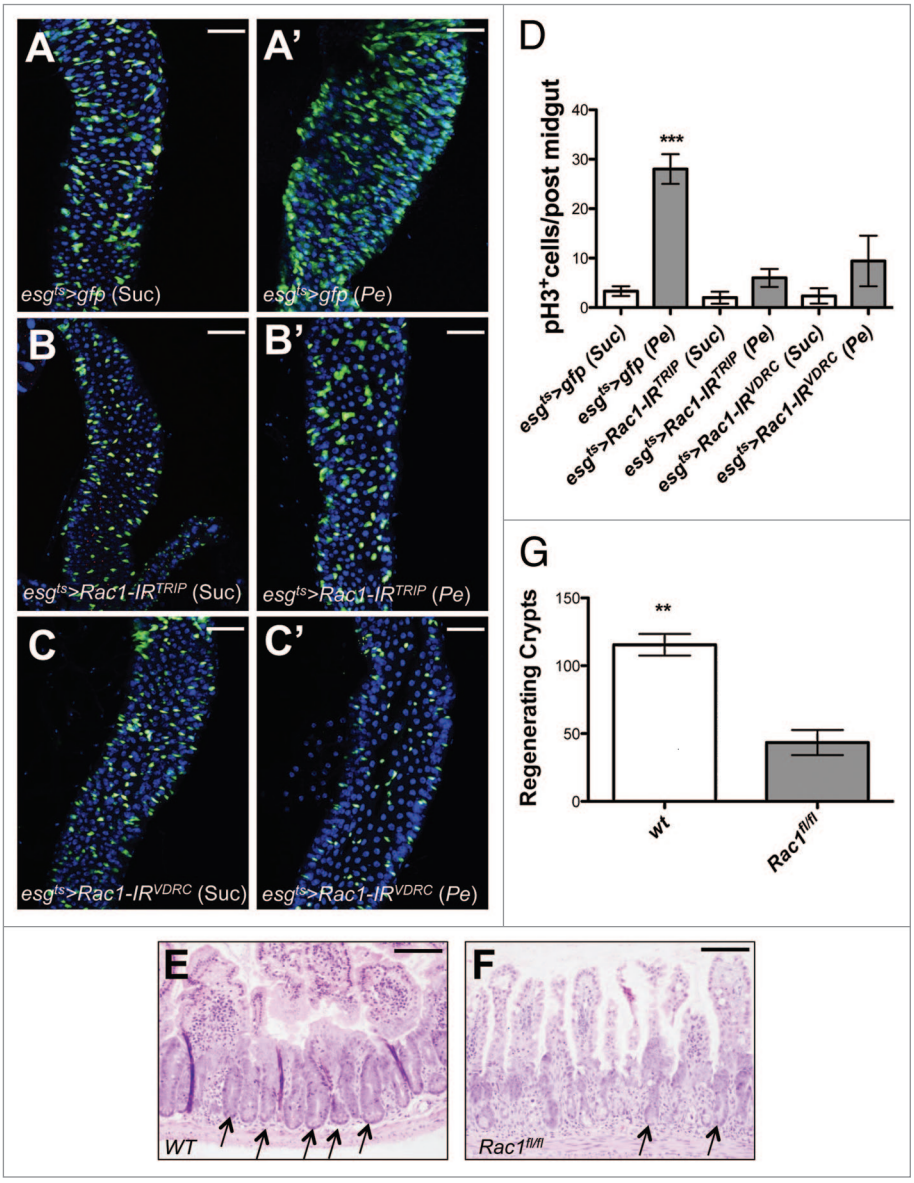

Figure 3. Rac1 is required for intestinal regeneration in *Drosophila* and mice. Posterior midgut from control animals (*esg*^{ts} > *gfp;* **A and A'**) or animals overexpressing 2 independent *Rac1* RNAi lines in ISCs/EBs for 10 d (*esg*^{ts} > *Rac1-IR;* **B–C'**) followed by Sucrose (**A, B, and C**) or *Pe* (**A', B', and C'**) feeding. Midguts were dissected and stained with anti-GFP (green) and DAPI (blue). (**D**) pH3 counts of posterior midguts as in (**A–C'**) (***$P < 0.0001$ one-way ANOVA with Bonferroni multiple comparison test). Scale bars: 50 μm. (**E and F**) Control (**E**) and *Rac1*-deficient mouse small intestines (**F**) subject to intestinal regeneration by DNA damage. Arrows point to regenerating crypts. Scale bars: 100 μm. (**G**) Quantification of the number of regenerating crypts in animals as in (**E and F**) (**$P = 0.0025$ unpaired *t* test).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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